

## ANTIBIOTIC SUSCEPTIBILITY TEST

Infectious diseases caused by pathogens are burden worldwide. Microbiological laboratory is mainly responsible for patient management and treatment including Isolation and identification of etiological agent and performing Antibiotic Susceptibility Test, thereby guide clinicians for respective therapy. Antimicrobial Susceptibility Test can help doctor to determine which drug can be more effective in treating the infection. There are a large number of Antimicrobial agents available for treating diseases caused by micro-organisms. These drugs are now essential part of modern medical practice, so there is need for judgmental use of these antibiotics. Antibiotic Susceptibility Test is measurement of the susceptibility of bacteria to antibiotics. Antibiotic Susceptibility Test may be done if infection does not respond to treatment. It helps doctor to find the most effective Antibiotic to kill an infecting micro-organism, pathogens such as bacteria, fungus etc. that invade our body and cause infection. The Antimicrobial agents used should aim at eliminating the infectious micro-organisms, so as to prevent the establishment of infection. It is possible for bacteria and other pathogens to mutate. Antibiotic that work today may not be effective after some years. Antibiotic Susceptibility Test specifies effective Antibiotic dosage and evaluating Antibiotic Resistance and helps in determining patient's treatment plans.

- This check technique is used as bacteria may develop resistant to some antibiotics.
- Drug Sensitivity Tests are important in study of the epidemiology of resistance and in studies of new antimicrobial agents.
- Antibiotic Susceptibility Test is applied to determine the susceptibility of pathogenic bacteria to antibiotics to be used in treatment.
- Antibiotic Susceptibility Tests are very useful for clinicians and hence constitute important routine procedure in diagnostic bacteriology.

### Uses Of Antibiotic Susceptibility Test:

- It is used to select effective drugs for treatment.
- It determines ability of the drug to kill bacteria.
- It may be used to figure out which antibiotic or combination of antibiotics will be most effective in treating the different types of bacteria causing the infection.
- It is used to identify organism if it has a characteristic sensitivity pattern.
- It is **NOT** indicated if sensitivity pattern of a pathogen cannot be predicted.
- Antibiotic Susceptibility Test is **NOT performed on commensals or contaminants.**
- This misleads physician and patient to receive necessary therapy.

- Such therapy leads to side-effects and is cause of emergence of resistant pathogens.
- It is used to determine MBC- Minimum Bacterial Concentration and MIC – Minimum Inhibitory Concentration of the Antimicrobial Agents used.

### **LIMITATIONS:**

- Antibiotic Susceptibility Test measures only In-vitro **Not In-vivo** drug activity.

### **Selection of best drug depends on:**

For therapeutic use, an Antimicrobial agent must exhibit Selective Toxicity. It must exhibit greater toxicity to the infecting pathogens than to the host. A drug that produces harmful effects or kills patients is of no use in treating infectious diseases even if it is lethal on micro-organisms.

Antibiotics represent a major class of antimicrobial agent. Antibiotics are biochemical produced by micro-organisms that inhibit growth or kills other micro-organisms. The discovery and use of Antibiotics have revolutionised medical field. But now organic chemists can synthesize the biochemical structures of many naturally occurring Antibiotics. Many antibiotics in current medical use are chemically modified forms of microbial biosynthetic products. Selection of best drug depends on:-

- Patient's clinical condition.
- Type and site of infection.
- History of drug hypersensitivity.
- Drug activity like absorption, diffusion in tissues, metabolism, excretion, toxicity, effect on patient's normal flora are **NOT** known by sensitivity testing.

### **ANTIBIOTIC SUSCEPTIBILITY TECHNIQUES:**

Antibiotic Susceptibility Technique has become concern worldwide as there is increase in Antibiotic Resistant forms due to indiscriminate use of Antibiotics in both man and animal. Antibiotic Susceptibility Test is useful tool to help quickly determine if bacteria are resistant to any drug. There are two methods of Antibiotic Susceptibility Techniques :-

- Diffusion Technique**
- Dilution Technique**

### **REQUIREMENTS for ANTIBIOTIC SUSCEPTIBILITY TECHNIQUES:**

AGAR SENSITIVITY TECHNIQUE:

- Mueller - Hinton Agar is used and considered best because Mueller Hinton Media is acceptable for batch reproducibility, for susceptibility testing, it is low in sulphonamides, trimethoprim and Tc inhibitors. It gives satisfactory growth of non-fastidious pathogens. It contains low concentration of Thymidine or Thymine.
- Other media that can be used are according to the nutritional requirement of test bacteria for example Lowenstein Jenson and Loefflers Serum Slope.
- Depth of medium should be 4mm (25 ml) in a sterilized petri plate.
- Pour sterilized media on plates on a level surface using Pour plate method in a sterilized petri plate.
- For spreading Inoculum Pour Plate or Swab Method is used.
- Too thin & too thick media gives false inhibition zones.
- Plates can be stored in plastic bags at 2 - 8°C for up to 2 weeks for later use.

#### DIFFUSION SENSITIVITY TECHNIQUE

- It is used in routine sensitivity testing.
- Inoculum gives semi-confluent growth.
- Filter paper discs 6mm in diameter are charged with required concentration of drugs and stored dry in the cold
- A disc of filter paper is impregnated with a known volume & concentration of a drug is placed on an agar medium inoculated with a test organism as explained above.

#### KIRBY - BAUER TECHNIQUE -

- Control organisms are also seeded in inoculated plate for effective comparison.
- Discs are placed so that Drug diffuses into the medium.
- After an overnight incubation, culture is examined for areas of no growth (inhibition zones) around discs.
- Sensitive bacteria are inhibited at a distance from disc.
- Resistant bacteria grow up to the edge of disc.
- Accordingly Antibiotics are classified as Sensitive, Intermediate and Resistant.

#### STOKES TECHNIQUE:

- A disc of filter paper is impregnated with a known volume & concentration of a drug is taken & placed on an agar medium inoculated with a test organism.
- Both test and control organisms are inoculated on same plate
- Disc is placed so that Drug diffuses into medium.
- Inhibition zone is compared directly with that of control. .

#### PLATING for Antibiotic Susceptibility Test:

- Using a sterile a 4mm loop, apply the organism suspension to center of sensitivity plate.
- Using a sterile swab, spread inoculum across the center third of the plate.
- Use of Control / Test is done.
- Do not use same swab for both application and spreading.
- For Inoculum - Check turbidity of inoculum with Mac Farland turbidity standard, not by naked eye.
- Similarly, inoculate the broth culture of control strain across the upper and lower thirds of plate, leaving 5 mm. on each side of test organism.
- Control suspension must be standardized against the standard.
- Allow the inocula to dry for few minutes, with the Petri dish closed.
- Place the antibiotic discs (after warming to room temp.) between test and control inoculum.
- Press disc down a little and do not move once it is placed.
- After ½ hr. incubate at 37°C. (For methicillin, incubate at 35°C)
- Read test when: Growth of both test and control strains is not too heavy or too light.
- Control inhibition zones measure 8-15 mm radius.
- Measurement is from edge of disc to edge of zone.
- If growth of test & control strains is not semi confluent, sensitivity test is repeated.

#### TURBIDITY (OPACITY) STANDARD

- ❑ Mac Farland Standard :  $1 \times 10^8$  CFU/ml

- It is a standard of Barium Chloride for matching turbidity of test & control strains inoculum.
- Add 1 ml of H<sub>2</sub>SO<sub>4</sub> to 99 ml water to make 1% H<sub>2</sub>SO<sub>4</sub>.
- Dissolve 2.35g Barium Chloride in 200 ml water.
- Mix 0.5 ml Barium Chloride solution to 99.5 ml H<sub>2</sub>SO<sub>4</sub> solution.
- Transfer turbid solution to screw-cap bottle of same type as that used to prepare test & control strain suspension.
- Turbidity standard can be stored in sealed container in dark, at room temp. for 6 months.
- Turbidity of standard is equivalent to an overnight broth culture.

#### ANTIMICROBIAL DISCS

- To select drugs for sensitivity, consult clinicians.
- Drug list must be limited & reviewed at regular intervals.\*
- If resistance developed, one member of each drug group is selected.
- Select Discs according to :
  - Control strains.
  - Select according to Site of infection in patient.
  - Drug concentration at this site.
  - Strain must respond to treatment with normal doses.
  - Strain must grow at same rate as test organism – when bacterial growth is heavy – zones are smaller and vice-versa.
  - Differences in molecular structure of drugs – larger zones are obtained when drugs diffuse rapidly in medium.

#### Recommended control strains

- *S. aureus* – Oxford strain - NCTC 6571
  - Used for all except polymyxins & for pathogens of all specimens except urine.
  - *E.coli* – NCTC 10418 - is used for all drugs against pathogens from urine.
  - *P. aeruginosa* – NCTC 13921- is used for controlling all drugs against *Pseudomonas*.

Precautions for control strains are:

- Strain is cultivated on Nutrient Agar slopes.
- Strains are stored in dark at room temperature. (20-28°C).
- Subculture is made every 3 – 6 months.
- Each week, a nutrient broth or agar culture is made & stored at 2 – 8°C & from this culture; suspensions are prepared for daily use.
- In Kirby-Bauer technique: zone is measured & compared against a previously prepared scale that correlates zone size with MIC.
- In Stokes technique: Inhibition zone is compared directly with that of control.

#### INTERPRETATION OF RESULTS

- Test is reported as: Sensitive, Intermediate & Resistant.
- Sensitive Zone: Test zone is wider than control zone or equal to control zone or not less than 3 mm smaller of control zone.
- Intermediate Zone: Test zone is more than 3 mm smaller of control zone but not less than 3 mm in diameter.
- Resistant Zone: Test zone is 2 mm or less or No zone of inhibition.
- Intermediate zone drugs must be prescribed in high doses to cure infection or when drug is concentrated at site of infection, e.g. UTI.
- With Sulphonamides & Trimethoprim, slight growth may occur within inhibition zone.

(This is due to presence of inhibitors (thymidine) and it must be ignored)

- Strains are considered resistant if:
  - Growth is heaped-up at zone edge without gradual fading up towards disc (penicillin-resistant Staph.)
  - Large colonies are seen growing within inhibition zone (Staph. aureus).
  - Colistin & Polymyxin give smaller zones of inhibition because of their large molecular size. (control zone must be at least 3-4mm)
  - Check your discs daily for any decrease in zone size resulting from drug deterioration.

## INHIBITION ZONES

- Inhibition zones vary in size due to:
  - Difference in molecular structures of drugs.
  - When bacterial growth is heavy.
  - Factors affecting the medium - volume, moisture, pH & constituents
  - Factors affecting the disc- drug concentration, storage & application.

## DILUTION SENSITIVITY TECHNIQUE

- Performed under conditions such as:
  - Patient not responding to therapy.
  - Patient is immunosuppressed.
- METHODS : 2 methods
  - Agar Dilution
  - Tube Dilution

## USES

- ❖ Measures the Minimum Bactericidal concentration (MBC)
- ❖ Measures the Minimum Inhibitory Concentration (MIC)
- It is useful in verifying adequate Drug concentration in blood and body fluids.
- It also guards us against excessive blood levels of toxic drugs.

## Technique:

- Dilutions of drug are added to a medium.
- A standard inoculum of organism is added.
- After overnight incubation, MIC is reported.
- Clinical response is assessed by comparing MIC obtained with already known concentrations of the drug.
- MBC may be determined by sub-culturing last tube in the dilution series to show visible growth.

- ❑ Other tubes should detect no growth on subculture.
- Dilution techniques require:
  - ✓ Good standardization
  - ✓ Good control of: inoculum, medium, drugs, incubation time, diluting techniques, reading of results.

MIC may be determined by automated machines.

- Before use, dry the plates with lids slightly open for ½ hr at 37°C.
- 5% blood is added to M-H agar to test for fastidious organisms (Neisseria, Haemophilus, Streptococcus).

Factors affecting Antibiotic Susceptibility Test

- Media containing substances inhibiting action of aminoglycosides, tetracyclines, trimethoprim, e.g: the substance thymidine.
- pH of media: False large zones are formed if medium is acidic (tetracycline), or false small zones if medium is alkaline (aminoglycosides).
- Fermentable sugars are not added to medium to avoid production of acid and change of pH.

Other factors include

- ✓ Manufacturer instructions regarding discs: store temp., expiry date, etc. are followed accordingly.
- ✓ Bring discs to room temp. (at least one hour before use)
- ✓ Do not expose discs to sunlight.
- ✓ Quality control of discs is essential.
- ✓ Avoid dryness & heat that decrease control zone size.
- SENSITIVITY TESTING:
  - Direct (Primary) test: Inoculum is a specimen.
  - Indirect (Secondary) test: Inoculum is a pure culture.
- METHODS OF INDIRECT TESTING:



- Apply Stokes technique as follows: Emulsify colonies of organism in Muller-Hinton broth.
- Match turbidity developed against standard turbidity.
- No incubation required.
- DIRECT SENSITIVITY TESTING:
  - Performed when Gram smear stain show large number of organism.
  - To obtain a presumptive result for serious cases.
  - For urine, pus, blood cultures.
  - If it is difficult to isolate & identify a pathogen or to detect a resistant strain.
- Sensitivity plate must not replace routine culture plate
  - Blood is added to Muller Hinton agar to be used for direct sensitivity.
  - Procedure for direct sensitivity is same as for indirect sensitivity.
  - Result of direct sensitivity must be confirmed by indirect sensitivity .
- Do not report direct sensitivity result if:
  - Growth of bacteria is too heavy or too light.
  - Zone size is smaller than that of the control.

#### Common Antibiotics in Use

- 1) Active against Gram Positive –
  - Penicillin (G & V)
  - Methicillin
  - Cloxacillin
  - Erythromycin
  - Novobiocin
  - Vancomycin
  - Bacitracin

- Fucidin

Active against Gram Negative –

- Polymyxin
- Aminoglycoside

3) Active against both Gram Positive & Gram Negative –

- Tetracycline
- Chloramphenicol
- Ampicillin
- Cephalosporins

Active against Fungi –

- Greseofulvin
- Iodides
- Nystatin
- Amphotericin B

### **LATEST TECHNIQUES: EMERGING TECHNIQUES**

Newer Antibiotic Sensitivity Test techniques, which are currently and actively being pursued by commercial entities for clinical translation, are considered as emerging technologies. With due increase of clinical demands for rapid Antibiotic Sensitivity Test, these techniques have been formulated. These techniques detect growth using cell lengths and numbers, forward light scattering, measuring vibrational amplitude changes of magnetic beads etc.. The resonators use nanoscale fluctuations. In these techniques Bio-markers or Bio- chemical markers use 16srRNA, ATP, Luciferase.

Many commercially available systems use automatic inoculating devices with multichannel pipettors. Broth micro dilutions results can be determined visually through automated instruments. Automation provides more precise, reliable and quantitative Antibiotic Sensitivity Test. These automated Antibiotic Sensitivity Test instruments require bacterial isolates obtained through routine culture from patients samples. Some of these instruments use photometer to detect turbidity over 4.5 to 18 hours to reveal Antibiotic Sensitivity Test results. However, in some Turbidometer has been replaced by colorimeter. Some instruments use Fluorescent

intensity monitored over 18-24 hours post incubation. Each of these instruments consists of the following-

- ✓ A single used AST cassette – micro-dilution tray – containing varied Antibiotics at different concentration
- ✓ An Antibiotic Sensitivity Test instrument which reads multiple cassettes over a period of time(usually overnight) to give Antibiotic Sensitivity Test results.

### **Latest Instruments**

- MicroShcan Walk Away - report ready in 3.5 – 7 hours
- Vitek 2 – Turbidimetric detection - report ready in 4 – 15 hours
- BD Phoenix automated System
- Sensiture system

### **Emerging Techniques**

- Optical Imaging
- Micro-Channel Resonators
- Biosensors
- Quantitating molecular or Biochemical markers

Imaging – based AST:

- Multiplexed automated digital microscopy (MADM) – uses Gel filters, electrokinetic loading and Fluorescent in-situ hybridized (FISH) probes – identifies bacterial cell in 1 hour – followed by AST
- Single cell morphological analysis (SCMA) – uses Bright Field microscopy
- oCelloscope

Non - Imaging – based AST:

- Forward laser light scattering – (FLIS)

Molecular Techniques:

Detection of Gene coding for resistance to one or several drugs by technique such as PCR and DNA hybridization

These approaches can significantly improve the current commercial Antibiotic Sensitivity Test technology but still rely on culturing, sample preparations, etc., and have some limitation.

Limitations:

- Its mechanism is limited to number of Antibiotics.
- It has limited concentration to be tested.
- Limited to analyzing Polymicrobial samples.